THE ISOLATION OF ACTIVE PRINCIPLES IN PURE FORM BY PARTITION*

LYMAN C. CRAIG

Professor and Member, The Rockefeller Institute

FAMILIAR words, like some of those in the title of this lecture, can communicate different ideas depending on the training and experience of the person to whom they are addressed. To a biochemist the term "active principle" usually refers to a single chemical compound, either simple or complex, but it may refer to a particular organization of chemical compounds, even to a living unit such as a virus or bacterium. Since the singular term "principle" rather than the plural is used, a direct relationship with the word "pure" is implied.

The term "pure", however, is considerably more difficult to define since it will vary greatly depending on the chemical nature of the active principle. If it refers to a single chemical compound, it is intended to mean that all, or practically all, the molecules in a given preparation are identical. If it refers to an organized complex, it means that other types of chemicals or complexes are absent as far as they are possible to detect. When more complex molecules or organized particles are involved, a solvent may be required to support the comparatively large and more or less extended mass. In this case one must deal with a solution and the solvent is considered only in the sense of a carrier or diluent. The word "pure", in the meaning to be used here, implies that we are able to detect to what degree the unwanted forms have been removed or excluded from a preparation.

From these few sentences it is obvious that the word "pure" in a way is an operational term. As used in classical organic chemistry, a preparation is pure when it can no longer be resolved into fractions with detectably different properties and always implies fractionation attempts with the most selective methods available. We shall bring out

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certain more critical factors bearing on the term "pure" as the lecture progresses.

The term "partition" usually refers to liquid-liquid extraction, a process based on equilibrating a solute with two immiscible or partially immiscible liquids. It can, however, have a much broader meaning and include the distribution of a solute between any two phases in intimate contact irrespective of whether true solution or adsorption is involved. The first more restricted meaning is considerably easier to interpret on a firm mathematical basis and will be used mostly in the treatment to follow. If the latter meaning should be used it would include most of the separation techniques employed today including chromatography.

Although liquid-liquid partition was widely used in the earliest chemical separations there is still much to learn about it. Only part of the influences that determine a partition ratio are known. From the enormous data accumulated thus far we can correlate gross differences in chemical properties with differences in partition behavior but often cannot use the information effectively for our purpose when the chemical differences are relatively small. It is inevitable that with the extension of detailed inquiry to larger and larger compounds or organized complexes, separations must be made on the basis of relatively small differences. Since we do not usually know what the differences really are, we must resort to an intelligent empirical approach, preferably one with the soundest possible basis.

With these considerations in mind, two different ways of making partition a more sensitive separation tool are open to improvement. One involves a search for solvents and conditions which will make a single partition more discriminating. The second involves a mechanical arrangement whereby larger numbers of single partitions (or their equivalent) may be made repetitively in countercurrent sequence so that the over-all discriminating power or selectivity may be increased We began intensive research along both these lines more than 20 years ago.

A countercurrent process may be accomplished either by a stepwise manner of contacting the two phases or by a continuous slow flow of the two phases countercurrently but in very intimate contact. The exact efficiency of the latter can be very high in terms of its over-all separating power, but the real basis of the discriminating power can be a matter of debate. Many comparisons of the continuous with the dis-

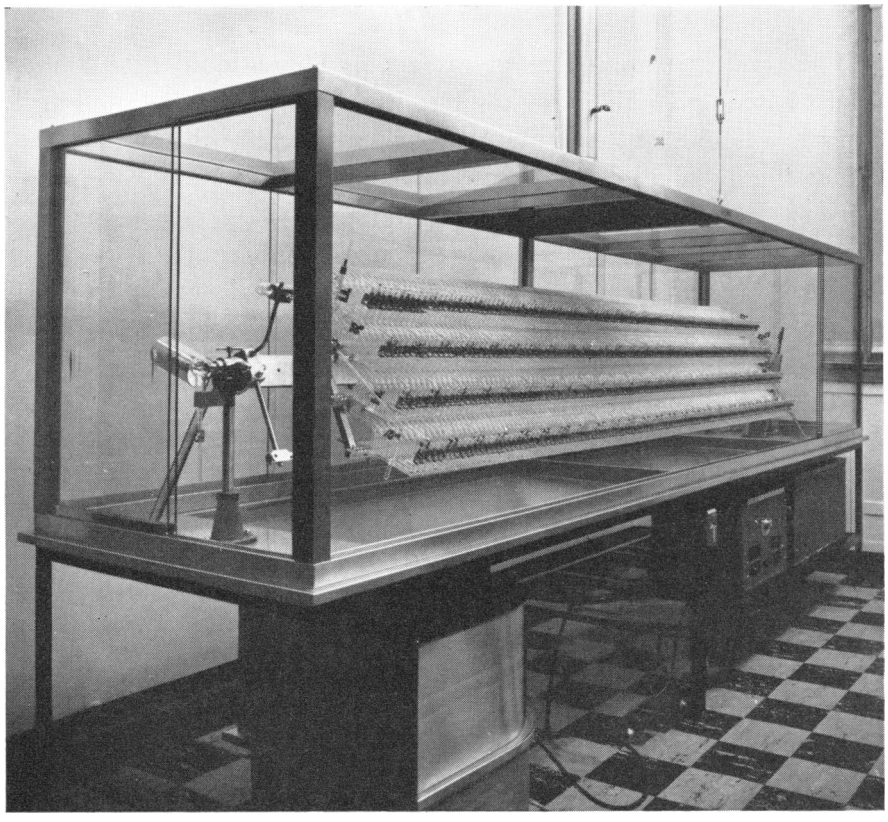

Fig. 1. A 1,000-cell automatic countercurrent distribution apparatus.

continuous process are to be found in the literature when the efficiency is obviously high and the calculated number of equivalent partitions large but few when it is mysteriously low, as is often the case with the isolation of a new active principle.

I shall not treat to any extent the continuous approach because it has been so widely used and discussed already in many previous award addresses dealing with the theory of chromatography. We have concentrated on the discontinuous approach with the thought that, in the end, it would contribute to a better understanding of separation as a whole and broaden the base of operation. There was also another cogent reason for pursuing the discontinuous approach. Because the continuous appeared to be easier experimentally and, in my opinion, theoretically oversimplified, hundreds of capable investigators all over

the world have been trying to improve it by every conceivable experimental variation, whereas relatively few have thought the discontinuous approach sufficiently promising to warrant devoting much time to it. Any scientific development worth while must meet and profit from vigorous competition. This can be very helpful in objectively evaluating a new approach. I shall treat of a number of cases in which the discontinuous approach has met the competition successfully and in doing so has brought certain problems of separation into sharper focus.

There will not be time to discuss the technical aspects which determine the design of our present partition equipment. Suffice it to say that trains of partition cells of different sizes and any desired length up to 1,000 cells have been developed and are available commercially. A considerable part of the credit for this must go to Mr. Otto Post who makes the equipment and has worked along with me throughout its development. The train with the greatest overall selectivity we have thus far built[1] is shown in Figure 1. It contains 1,000 partition cells and is fully automatic with a filling device and fraction collector.

This type of train performs serial partitions in countercurrent manner so that the mathematics of the binomial theorem can be directly applied to fractionation. An orderly distribution of fractions throughout the train is thus obtained which is amazingly reproducible. We have given the name "countercurrent distribution" to such a process, CCD for short. One concept of the capability of the apparatus is shown in Figure 2. It is a plot showing distribution patterns calculated for 28 hypothetical compounds with appropriately chosen partition ratios at 4,000 transfers or cycles. At this point 3.5 million separate partitions have been made, 1,000 for each transfer after the first 1,000. In this pattern the fraction of substance in the original mixture, or a figure proportional to it, is plotted against the serial number of the partition cell. Each separate peak can be calculated by the equation for the normal curve of error which in fact happens to be an approximation permitting an extension of the binomial theorem to high numbers of cases.

We can say from this pattern that the mechanical aspects of the process will permit us to separate in good yield the different members of a family of substances whose partition ratios differ from each other by a factor of 1.2, or a considerably smaller factor if part of the yield is sacrificed. Other variations in the procedure, fully described elsewhere[2] but too technical to discuss here in the time allowed, also

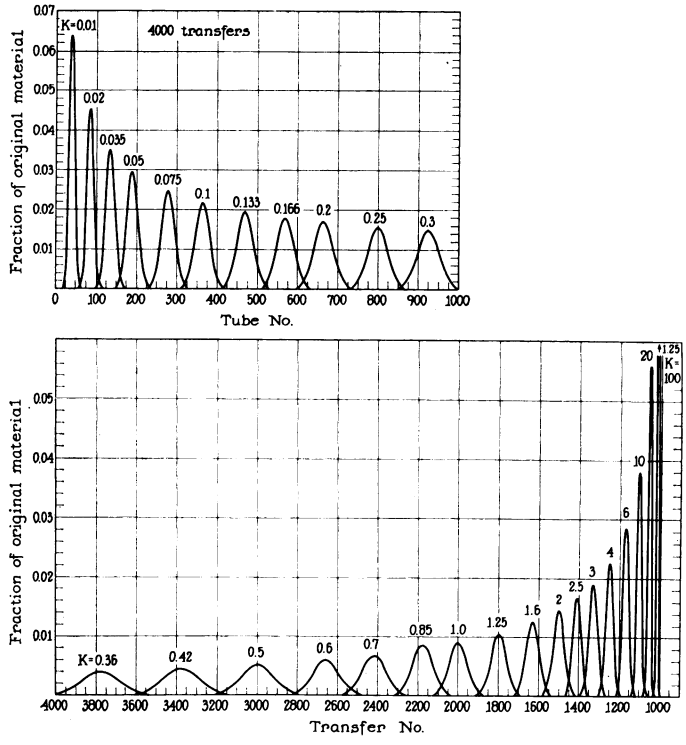

Fig. 2. Calculated CCD patterns for various partition ratios at 4,000 transfers. Upper pattern = fractions remaining in the train. Lower pattern = effluent fractions.

increase the over-all discriminating power.

This is a very brief mention of the mechanical aspects of CCD which has been intended to show something of its potential as well as its limitations. We shall now turn our attention to the choice of two liquid phases which may give a selectivity sufficient to be effective in a train of the length just mentioned. We call the two liquid phases a "system". The system is, of course, the real key to any separation problem. The mechanical aspects at best only extend the range of possibility offered by the system.

A curious thing about simple partition is the fact that it sometimes shows very high inherent selectivity, and a distribution train then is scarcely needed or, at most, only a very short one. More than 20 years ago it was discovered that very high selectivities could be obtained in separating amines when one of the two phases was a concentrated buffer.[3] This was so effective that even a short train with 25 partition

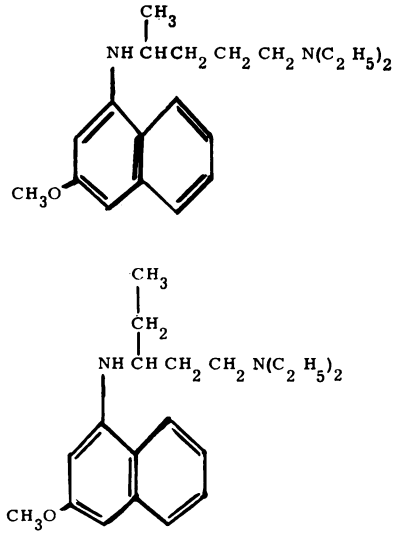

Fig. 3. Structural formulas for plasmochin (upper) and its isomer (lower).

cells became the tool of choice for purifying the much needed synthetic antimalarials during the war and for proving purity. The problem was often that of separating isomers[4] such as shown in Figure 3. The method was also effective in isolating the metabolic transformation products of the antimalarials from body fluids.[5] No other separation tool was as effective. The same type of buffer was later found effective in chromatography.

At the close of the last war an interesting problem in the study of the purity of another class of very important active principles arose. Many preparations of the wonderful new antibiotic, penicillin, did not seem to have the original effectiveness and there was much speculation that the pathogens were becoming resistant due to mutations. It so happened that the commercial preparations were lyophilized residues purified by various manipulations to the highest antibiotic activity. Other data to show that they were pure chemical individuals were lacking. Dr. George Hogeboom and I, in collaboration with Drs. Carpenter and du Vigneaud, had used the concentrated buffer[6] systems and found we could readily separate the various types of penicillin by CCD. We further found that the current commercial preparations contained little of the penicillin G which had been so effective in the initial clinical

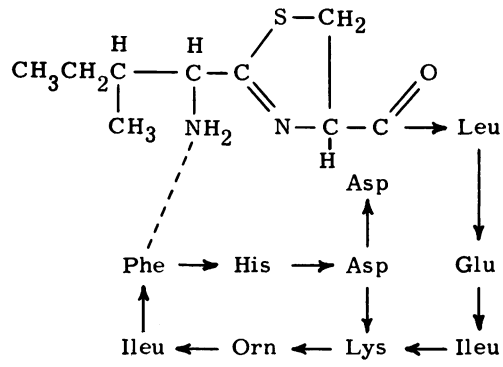

Fig. 4. Structural formula of bacitracin.

trials, something not realized by the manufacturers or the clinicians. Instead, a different penicillin type of high antibiotic activity, but one quickly eliminated from the body, was found. When this was discovered the manufacturers changed their process so that crystalline penicillin G was produced and the practical therapeutic problem with the new drug was solved. Following this experience, CCD became one of the major tools used in documenting purity with the international standard sample of penicillin G.

I would be remiss if I did not mention the studies of Professor du Vigneaud[7] and his collaborators during the war concerning the structure of penicillin, particularly that which led to its synthesis. One of the points of their work pertinent to this lecture is that they used CCD for the purification of the complicated mixture resulting from the difficult synthesis and obtained pure crystalline penicillin G synthetically for the first time. All other methods of purification, including chromatography in many other laboratories, had not succeeded.

Unfortunately, in further investigations the concentrated buffer systems failed to provide high selectivity for amphoteric highly water-soluble substances such as the amino acids and many peptides. It was, in part, for this reason that our efforts were directed toward the development of machinery capable of providing high numbers of transfers. Another reason was the finding that some of the factors most important with small molecules were not decisive with the larger ones. It was the challenge of the larger more complicated active principles that really held the greatest interest for us.

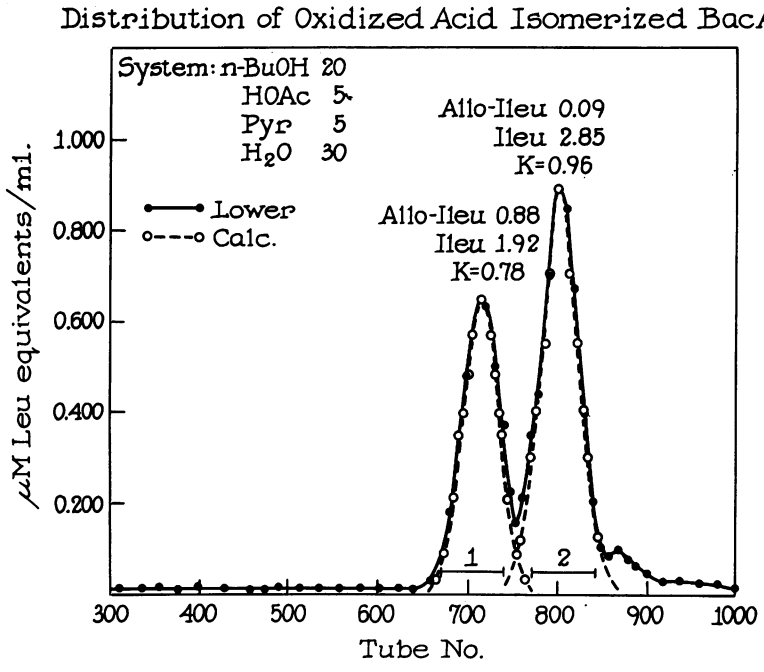

Fig. 5. Countercurrent distribution pattern of the separation isomers of oxidized bacitracin A.

At that time, scarcely more than ten years ago, detailed chemical structures for molecules with molecular weights larger than 1,000 were practically unknown. This meant that the separation studies had to be applied first to substances of unknown structure. It was therefore of the highest importance to carry out detailed structural studies concurrently as an integral part of the program.

One model we chose for the purpose was a new promising antibiotic, bacitracin, discovered by Johnson, Anker and Meleney.[8] It was thought to be about five- to ten-fold the size of penicillin. Our methods of investigation, which relied heavily on partition, steadily afforded results and in due time[9] we were able to write the formula given in Figure 4. It was also derived independently by Lockhart, Abraham and Newton[10] using similar methods. Our more recent unpublished data have shown that it is a tightly packed almost spherical molecule held together in this conformation by a combination of secondary forces and labile covalent linkages.

In spite of this advanced stage of the work, our attempts to prove final purity were constantly thwarted by variable antibiotic activity assays. Finally, after stabilizing the molecule by oxidizing the sulfur we were able to separate cleanly two isomers[11] as shown in Figure 5. The only difference between the two isomers was in the optical activity of the alpha carbon atom of the amino terminal isoleucine. It was found to racemize easily in the intact antibiotic. This seems a small difference when a molecular weight of 1,422 is considered.

As our work progressed we naturally followed with the greatest interest the well-known pioneering work of Professor du Vigneaud and collaborators on the isolation and proof of structure of oxytocin and vasopressin.[12] Since they made such effective use of the CCD procedures, their success gave us valuable encouragement, particularly during those times when our own progress was dragging. Of the many striking separations they have published, I shall mention only one which seems particularly significant. They have recently synthesized an isomer of oxytocin in which the levorotatory leucine residue is replaced by the dextrorotatory form.[13] This would not seem to be much of a difference in a molecule the size of oxytocin, yet they found CCD would readily separate a mixture of the natural hormone and the synthetic isomer.

About 15 years ago we needed a protein for partition studies in the molecular weight range of 10,000-15,000 and selected insulin as a model. Reliable data had been published by physical methods[14] to indicate a value of 12,000. We were soon able to develop a system for it[15] and to demonstrate separating potential by removing a form of insulin in which one of the six amide groups was not present, a rather small difference. The correct amino acid composition was established for the first time on such a preparation by Harfenist.[16]

We also prepared dinitrophenyl derivatives in which the basic groups were only partially covered, and separated them by CCD. From data obtained in this way and by spectroscopic characterization, not only was the purity confirmed but the true molecular weight of 6,000 instead of 12,000 also was derived conclusively.[17] These data were used by Sanger in establishing the sequential formula of insulin.

In last year's Lasker Award address, Dr. C. H. Li gave a very impressive review of his work and that of others with the polypeptide hormones of the anterior pituitary. We had followed with great interest and admiration the emergence of these data through the years for each

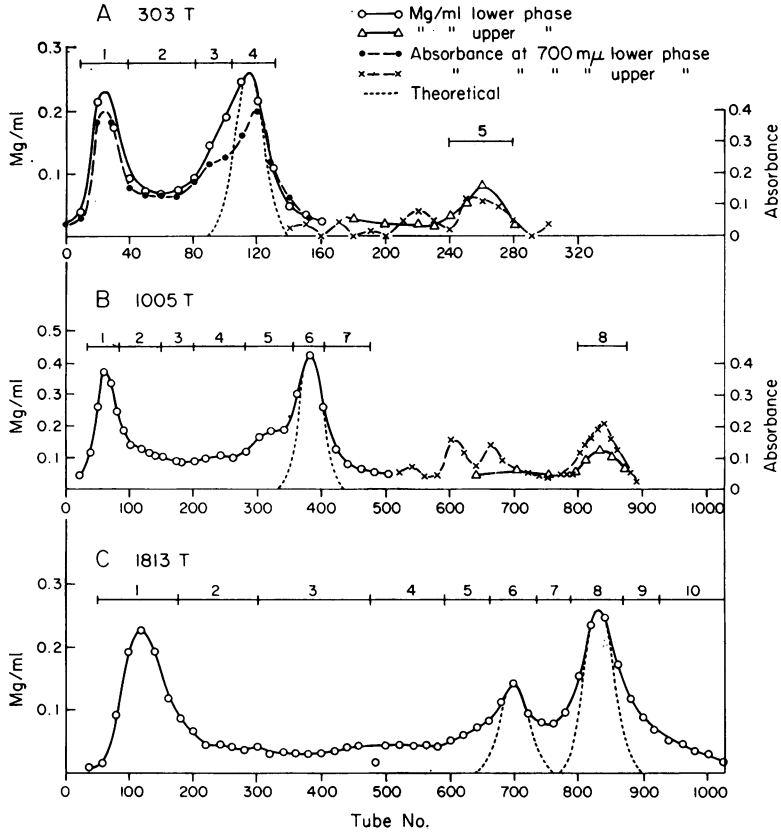

Fig. 6. Countercurrent distribution patterns of crude A.C.T.H. at 303, 1,005 and 1,833 transfers respectively.

hormone, but particularly that with ACTH coming from three different laboratories,[18-20] Dr. Li's group, Dr. Bell's group and Dr. White's group. Recently, after the sequence problem was considered settled, we were stimulated to reinvestigate the isolation of pure ACTH by CCD. There were several reasons for again taking up the study. A paper was published recently[21] in which various hormones purified by CCD were claimed to be impure by starch gel electrophoresis. Our main reason, however, was that we needed this well-documented peptide with 39 amino acid residues to use as a model in conformation studies with a membrane diffusion method we are developing.[22]

A sample of crude ACTH was fractionated by CCD as shown by the patterns at various numbers of transfers in Figure 6. Hormone isolated

from the largest peak gave an analysis by the Spackman, Stein and Moore chromatographic technique[23] in complete agreement in every way with that of a pure peptide with the published amino acid composition.[19] The value of such analyses and the method in establishing a practical degree of purity is beyond question. This material gave a biological assay comparable to the best preparations and was homogeneous by the membrane dialysis technique.[22] T. P. King found it to give a single well-defined spot by the starch gel electrophoresis technique, thus removing the implication[21] that ACTH had not yet been prepared in pure form.

In membrane diffusion studies with this preparation and with other similar peptides, evidence too involved to discuss here was obtained showing that their conformation in solution, i.e., their shape, is extraordinarily sensitive to the solvent environment or temperature and that they rapidly, if not instantly, adjust to a change in the environment. This contrasts with the behavior of proteins such as ribonuclease which are cross-linked by s-s bonds. Ribonuclease, M.W. 13,600, is an enzyme whose amino acid sequence has been entirely elucidated by Hirs, Moore, Stein and their collaborators.[23, 24] In the ACTH type of protein the phenomenon of denaturation is less of a problem in fractionation since here the conformation seems to be almost completely mobile. Thus in CCD the effective partition ratio could be determined in large part by the conformational mobility of the molecule. The conformation would be very different in the organic phase from that in the aqueous phase and selectivity could be due to an environment providing precisely the right balance of conformational averages.

It is interesting to compare this behavior with the distribution behavior of ribonuclease which can be precisely fractionated by CCD, but most favorably in a system of high salt content. A family of closely related ribonucleases has been fractionated by Eaker[25] as shown in Figure 7. The separation has also been cross-checked by ion exchange chromatography. The largest band is ribonuclease A, the second is ribonuclease A with the amino-terminal lysine removed and the third differs from the second by having lost a single molecule of water from the amino-terminal glutamic acid residue. The separation is of considerable interest as a purity study.

Even more interesting from this standpoint was the finding[26] that ribonuclease A, purified by ion exchange chromatography and recov-

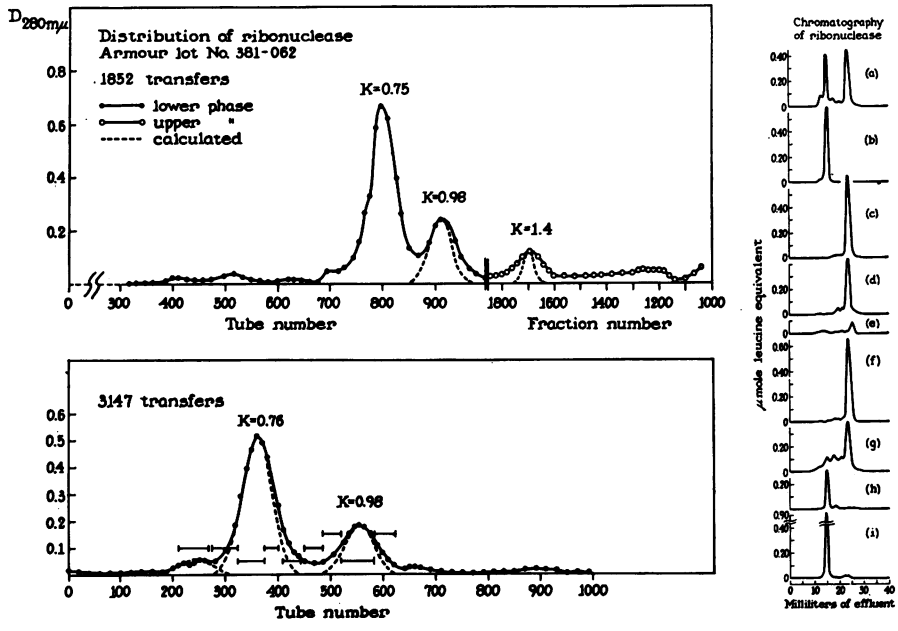

Fig. 7. Countercurrent distribution patterns of a ribonuclease sample.

ered as a lyophilized residue under certain conditions, showed a different behavior in CCD than had previously been encountered.[25] The pattern at 1,050 transfers indicated impurity as shown in Figure 8, but the supposed impurity failed to separate as it should have done at 4,012 transfers. Instead, it was indicated that a shift in partition ratio was slowly taking place in the system during the fractionation. Membrane diffusion studies confirmed the theory of a slightly different conformational form, metastable in the CCD system.

If the present-day concepts of protein structure concerning intrachain interaction and the phenomenon of denaturation are correct, it could have been predicted that, with the development of a mild yet sufficiently selective separation method, we would inevitably encounter the problem of this type of isomerization. In any case, such a likelihood is a point to consider in our ultimate definition of purity since it has now been demonstrated in ribonuclease and indicated in other proteins as well.

A closely related problem is that of intermolecular interaction and association to reversible polymers. Obviously, the separations mentioned

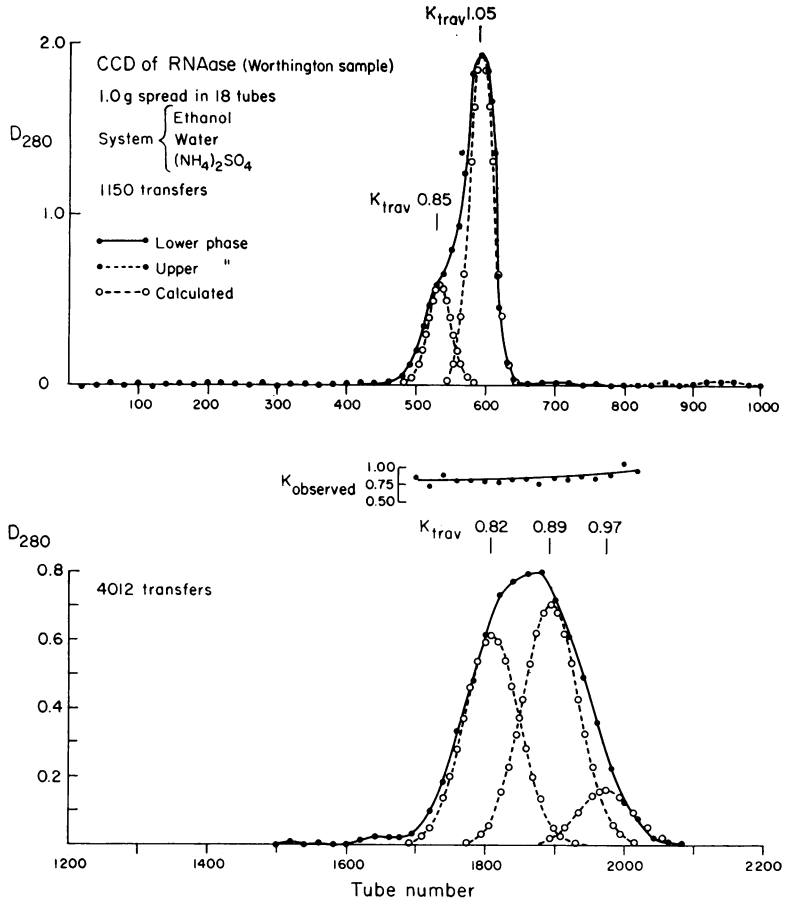

Fig. 8. Countercurrent distribution pattern of a ribonuclease sample undergoing a transition.

earlier in connection with insulin have dealt with this problem. Insulin associates strongly to varying degrees depending on the pH and environment. At pH 3 it exists chiefly as the dimer with a molecular weight in the 12,000 range. Yet the dinitrophenyl derivatives showed sufficient dissociation in the CCD system so that the true monomer was clearly revealed.

Another example connected with the isolation of oxytocin was found. Oxytocin in the gland occurs specifically bound with a larger polypeptide component of the pituitary gland which has been given the name "neurophysin".[27] The system used for CCD causes sufficient

dissociation so that the repetitive partition will completely remove the hormone from the components to which it is bound. A similar phenomenon may occur in the isolation of ACTH and other active principles.

The most recent and highly documented example of a specific type of association is to be found in hemoglobin. Here two different types of protein chains and four hemes associate in a specific way to form a tetramer. The complex can be dissociated by stages, first by partition in a mixture of dilute acid and methyl ethyl ketone to remove the heme. The best method of separating the two protein chains remaining in the globin is by CCD in an appropriately dissociating system, one of nearly the same composition as that found effective with insulin. In this way pure individual chains were provided in the quantity which permitted the entire amino acid sequence of each chain to be determined.[28] One chain contains 141 amino acids, the other 142.

If it is desired to isolate the intact hemoglobin complex, a system with less dissociating properties would be required. The one containing considerable ammonium sulfate developed for ribonuclease should be nearer this type. When tried with intact hemoglobin only one band was found, but there was still evidence of partial removal of the heme. The alpha and beta chains, however, remained together and were not separated.

The mildest systems from the standpoint of denaturation have been developed by Albertsson[29] at Uppsala, Sweden. They are made from aqueous buffers and polymers such as the polyethylene glycols and dextrans. These interesting systems, containing mostly water, separate into two phases only very slowly, but when they do they show surprising selectivity for large organized complexes such as viruses and cell particles. Even closely related strains of bacteria have been separated,[30] as shown in Figure 9, by CCD using these systems.

A number of very extensive studies have been touched upon here only very briefly. They have been presented to show that the key to separating large molecules by partition may lie in the choice of systems which have a mild denaturing or dissociating effect on the solute which is barely sufficient to permit the repetitive effect of CCD to achieve the separation. Too strong a dissociating effect may bring about irreversible denaturation. By choice of suitable solvent components the dissociating effect may be titrated so delicately that organ-

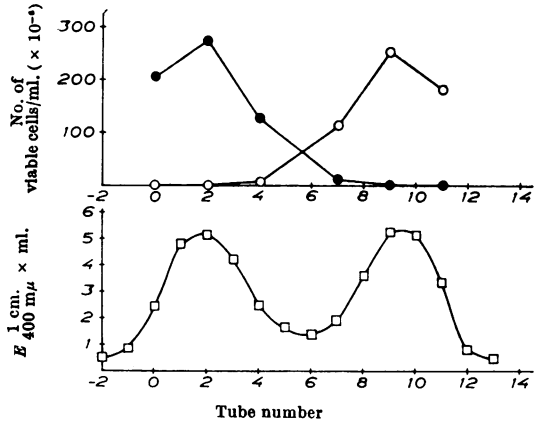

Fig. 9. Countercurrent distribution pattern of a mixture of two strains of *E. coli*. The pattern at the top shows viable counts in each tube, that below shows the amount of bacteria expressed in extinction values times the volume of phase system in each tube.

ized complexes may be separated from each other, even without disrupting the organization.

After the foregoing discussion we are in a position to treat more intelligently a problem which often occurs in the isolation of active principles from tissue. Enzymes capable of transforming the principle are likely to be present. Their action is normally controlled in a variety of ways, including segregation by membranes, by the presence of inhibitors, by the physical state of the active principle, i.e., its conformation, state of association and the degree to which it is bound to other tissue components. When the organization is disrupted by mincing, grinding and extracting the tissue, the action of the enzymes may be considerably enhanced. Many times, the activity known to be originally present mysteriously disappears as the work progresses or the yield is variable with repeated experiments. The ACTH type of peptide, for instance, with its flexible conformation is particularly susceptible to hydrolytic enzymes.

A good example of the discouraging problems involved, some of which are probably due to the aforementioned state of affairs, is to be found in the experiments leading to the recent isolation of pure parathyroid hormone. Rasmussen[31] had overcome many difficulties and had obtained the highest activity yet reported by a variety of fraction

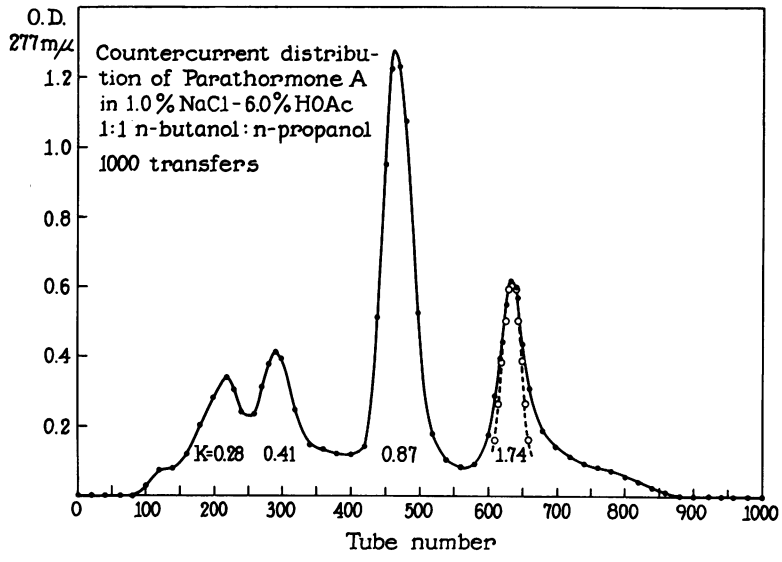

Fig. 10. Separation of parathyroid hormones.

techniques. However, when CCD was tried (Figure 10), the preparation was resolved into at least four components only one of which, K = 1.74, was active. The activity of this material was much higher than any yet isolated. The material used in the CCD experiment had been obtained by dilute hydrochloric acid extraction. It had poor stability and proved to have a molecular weight of 3,800.

In further experiments, however, it was found that if the active hormone was extracted from the tissue with phenol and fractionated by CCD a more stable product resulted[32] and the molecular weight was more than double that obtained by the dilute hydrochloric acid extraction. Other intermediate sizes were obtained depending on the extraction procedure. While there was no direct evidence to show that enzymatic action was directly involved in the variability, it was one possibility to consider.

Success thus may depend on getting the ground tissue quickly into a system with a critical degree of dissociating power, one strong enough to inhibit all enzymatic action by denaturation, and at the same time bring the active principle into a physical state favorable to separation by partition. Phenol is a powerful dissociating agent. The trichloroace-

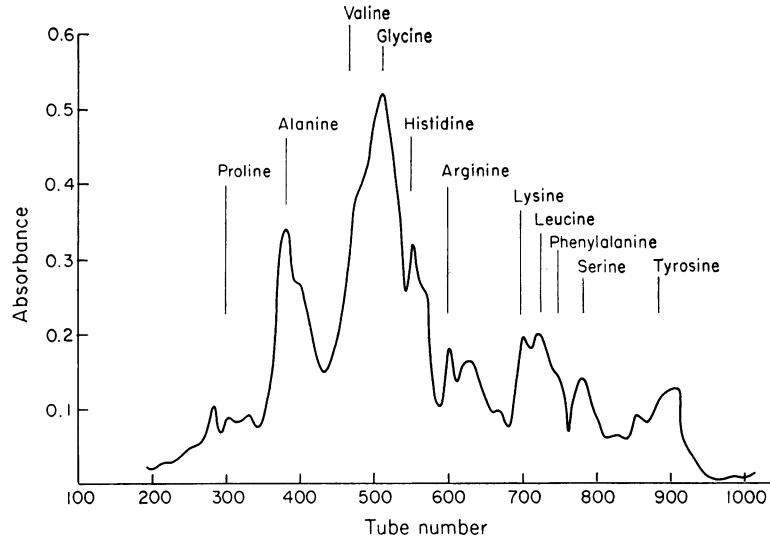

Fig. 11. Countercurrent distribution pattern of Coli S-RNA at 1,000 transfers.

tic acid systems, the pyridine systems, etc., have been found useful in the isolation of hormones from many sources.

From the separation standpoint, one of the major problems facing biochemistry today is the problem of the nucleic acids. We have, as yet, no satisfactory way of separating the individual members of this important group and therefore cannot speak of a pure nucleic acid in the sense of purity implied by this lecture. A good start in this direction, however, has been made with the S-RNA types by use of CCD in Holley's laboratory at Cornell University.[33] This type is the smallest of the nucleic acids. It consists of mixtures of polynucleotides, each containing on the order of 100 nucleotides. There are at least 20 of the individual chains to be separated, one specific for each amino acid. Systems containing much phosphate, formamide, propyl alcohol and water seem the most effective. One of the recent separations obtained by Dr. Goldstein in my laboratory is shown in Figure 11. The reason for the wide range of partition ratio extending from 0.25 to 10 is an intriguing problem. Undoubtedly, there must be very delicately balanced dissociating and conformational effects controlling this separation. At the present time we are in need of a better way of recognizing and defining these effects.

The difficulty indicates that proper characterization of such labile and complicated substances as the nucleic acids will require a higher order of over-all selectivity than we can now apply. It has been our hope that some of the considerations briefly discussed in this lecture will point the way toward the development of this selectivity.

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